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# Cell surface protein partially restores morphology, adhesiveness, and contact inhibition of movement to transformed fibroblasts

(cell adhesion/transformation/3′:5′-cyclic AMP/growth control)

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ABSTRACT **We have isolated the major cell surface glycoprotein of chick embryo fibroblasts, CSP, and added it to a variety of transformed cells *in vitro*. The transformed cells become more elongated, often more flattened, and show increased adhesion to the substratum. Several transformed cell lines also align in striking parallel arrays. This alignment is characterized by a decrease in the amount of nuclear overlapping, probably indicating restoration of contact inhibition of movement. The morphological changes are antagonized by antibody to CSP. These effects of CSP are not associated with an elevation of cellular 3′:5′-cyclic AMP. Moreover, the morphological reversion is not accompanied by an alteration in growth properties. Our results are consistent with a role for CSP in cell adhesion and morphology but not in growth control.**

The major cell surface glycoprotein of chick embryo fibroblasts, CSP, is substantially decreased after transformation by oncogenic viruses. Similar proteins, designated "LETS" proteins, are decreased in other cell types after transformation (refs. 1–3; for other references see 4 and 5). We have isolated CSP and found that it will aggregate several cell types, including erythrocytes, embryonic chick cells, and transformed NRK (normal rat kidney) cells (6, 7), suggesting that CSP may play a physiological role in cell:cell adhesion. The agglutinin activity of CSP is destroyed by treatment with proteases and chelating agents and is greatly diminished in transformed chick cells (7). Since transformation is often accompanied by altered growth control, morphology, adhesion, and contact inhibition of movement, we investigated whether re-attaching this glycoprotein to the cell surface could restore a more normal phenotype to transformed cells. We find that CSP partially restores morphology, adhesion, and the alignment characteristic of untransformed fibroblasts.

## MATERIALS AND METHODS

**Isolation of CSP.** We prepared CSP from secondary cultures of chick embryo fibroblasts as described previously; the protein is essentially homogeneous by electrophoresis in sodium dodecyl sulfate with or without 8 M urea at pH 7 or pH 11 (ref. 7; unpublished). We then added solid ammonium sulfate to 70% saturation and adjusted the pH to 7.4 with $NH_4OH$. After 30 min at 4°, the solution was centrifuged at 25,000 × *g* for 15 min. The pellet was solubilized in 1/20 volume of buffer A (0.15 M NaCl, 1 mM $CaCl_2$, 10 mM cyclohexylaminopropane sulfonic acid, pH 11.0) at about 1 mg/ml, dialyzed overnight against two changes of 400 volumes of buffer A, and stored at −70°. These conditions are optimal for maintaining a concentrated, nonaggregated preparation of CSP with high agglutinating activity (unpublished data); this solution was neutralized with HCl just prior to addition to cells.

**Cell Culture.** Cells were passaged with 0.25% trypsin and plated at $2.5 \times 10^5$ per 35 mm tissue culture dish (Falcon) in a final volume of 1 or 1.5 ml of Dulbecco-Vogt modified Eagle's medium containing 10% calf serum (Colorado Serum; inactivated for 30 min at 60°). Where indicated, we added CSP (42–50 μl/ml) to give a final concentration of 50 μg/ml of protein (8). Controls received an equal volume of medium, saline, or buffer A. Cultures were fed daily with control or CSP-containing medium.

**Adhesion.** SVT2 cells (Balb/c 3T3 mouse cells transformed by simian virus 40) were plated at $2.5 \times 10^5$ per 35 mm dish and cultured in medium with or without 50 μg/ml of CSP for 24 hr. The cells were gently rinsed twice with Dulbecco's calcium-magnesium-free phosphate-buffered saline, then agitated in 1 ml at 200 rpm on a rotary shaker (New Brunswick Scientific model G-2) for 5 or 10 min at 37°. The detached cells were aspirated; these cells and those remaining on the substratum were dissociated with 0.25% trypsin and counted with a Coulter counter to determine the percent cells detached.

**Cytoplasmic Area and Nuclear Overlap Ratio.** SVT2 cells were plated at $1 \times 10^4$ per $cm^2$ on glass coverslips in CSP-containing or control media. After 48 hr they were fixed and stained with hematoxylin (9). Cytoplasmic area was measured using the intersections of a 10 × 10 microscope eyepiece grid by the formula $A = aCF/IN$, where $A$ = mean cytoplasmic area, $a$ = area of the slide covered by the grid, $C$ = number of intersections superimposed on cells, $F$ = number of fields counted, $I$ = number of intersections, and $N$ = total number of cells.

Nuclear overlap ratio was determined by Weston's modification of the method of Abercrombie (9), substituting the 10 × 10 eyepiece grid for the Chalkley array.

## RESULTS

**Morphological Effects of CSP.** Purified CSP significantly altered the morphology of all nine transformed cell lines tested (Fig. 1, Table 1). Controls receiving buffer A showed no morphological changes. The morphological alterations induced by CSP included cell flattening, elongation of processes, and parallel alignment of cells, generally resulting in a more fibroblastic appearance. After 8 hr with CSP, virtually all the SVT2 cells appear flatter and more elongated (Fig. 1a and b). By 24 hr the cells are more bipolar and begin to align in parallel arrays, which become more prominent as the cells become denser (Fig. 1c, d, and f). Flattening and alignment occurs at concentrations of CSP as low as 1 μg/ml. These morphological effects also occur with a CSP prep-

Abbreviations: NRK, normal rat kidney cells; SVT2, Balb/c 3T3 mouse cells transformed by simian virus 40; buffer A, 0.15 M NaCl, 1 mM $CaCl_2$, 10 mM cyclohexylaminopropane sulfonic acid, pH 11.0.

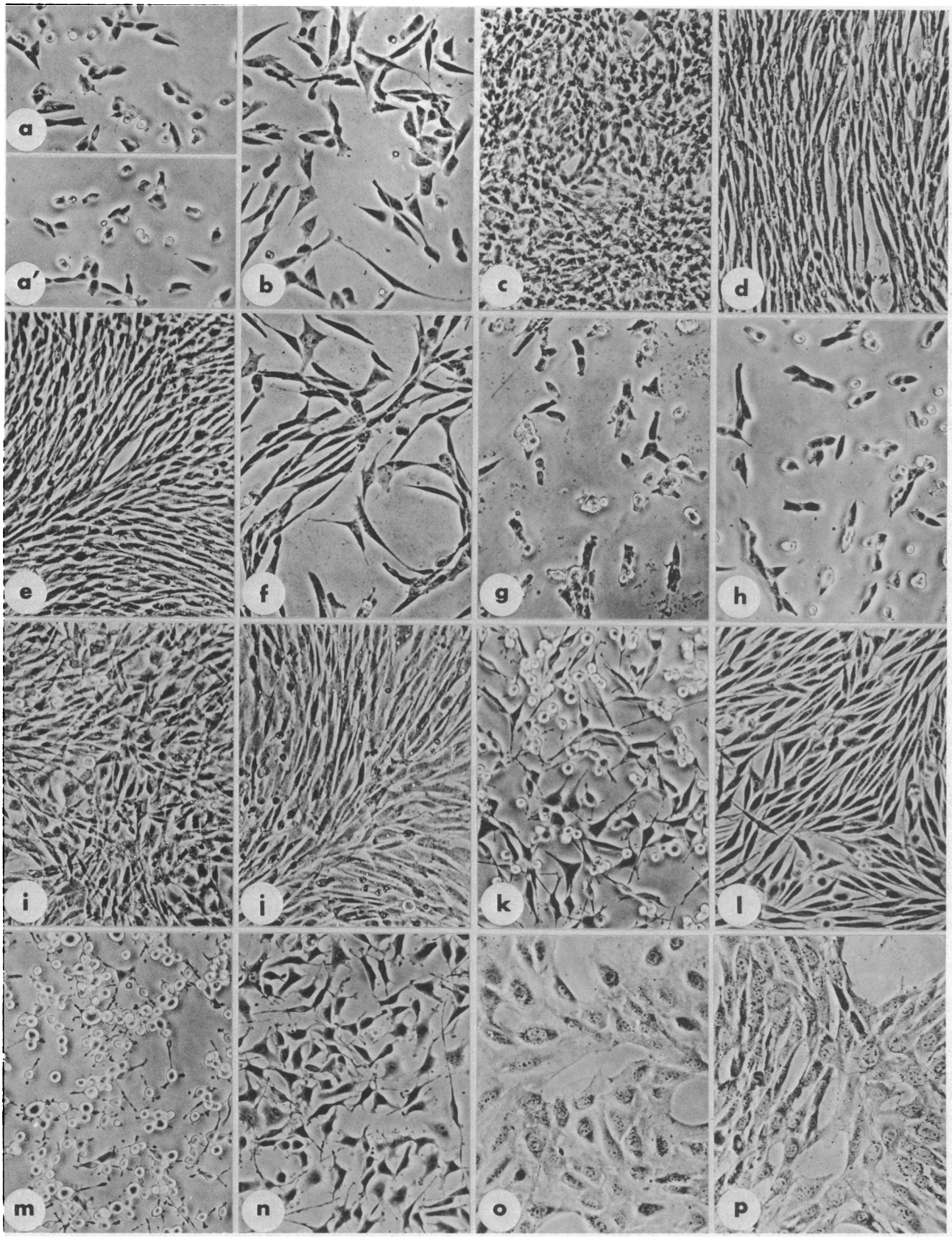


FIG. 1. Effects of isolated CSP on the morphology of transformed and untransformed cells *in vitro*. All CSP additions were at 50 μg/ml of culture medium, and the buffer A additions consisted of the appropriate CSP diffusates. SVT2 cells 8 hr after plating in regular culture medium (a), in medium containing buffer A (a'), or in medium plus CSP (b). SVT2 cells 72 hr after plating in medium plus buffer A (c) or plus CSP (d). SVT2 after 48 hr with CSP that had been affinity purified using erythrocytes and chromatographed on Sephadex G-200 (e).

*(Legend continued at bottom of next page)*

Table 1. Effects of purified CSP on cell morphology

| | Flattening | Cell process elongation |
|---|---|---|
| SV40* Balb/c 3T3 | ++ | ++ (++) |
| Schmidt-Ruppin RSV* chick embryo cells | ++ | ++ (++) |
| Kirsten sarcoma virus NRK | ++ | ++ (++) |
| Moloney sarcoma virus NRK | + | + |
| Harvey sarcoma virus NRK | ++ | + |
| L 929 mouse cells | ++ | + |
| Schmidt-Ruppin RSV NRK | + | + |
| Bryan high titer RSV chick embryo cells | − | + |
| SV40 WI38 human cells | − | + |
| Balb/c 3T3 mouse cells | − | + |
| NRK | − | + |

Parentheses indicate concomitant alignment of cell bodies. Each cell type was incubated for 3 days in 50 μg/ml of CSP or control medium and examined and photographed daily. ++ indicates a striking morphological response; + indicates a definite response; and − indicates a questionable or absent response. See Fig. 1 for examples.

* RSV, Rous sarcoma virus; SV40, simian virus 40.

aration (7) not fractionated by ammonium sulfate. Furthermore, CSP purified by absorption to and elution from sheep erythrocytes (7), and chromatographed on a Sephadex G-200 column at pH 11 in buffer A, also induces these morphological effects on SVT2 cells (Fig. 1e). CSP also acts when added to cells 24 hr after plating.

Specific antibody to CSP antagonizes the morphological effects (Fig. 1f, g, and h); a 1:15 dilution of the globulin fraction (see ref. 7) completely antagonizes 50 μg of CSP. This concentration of antibody does not affect the morphology of SVT2 cells in control medium, and control goat antiserum (7) does not antagonize the CSP effect. Treatment of untransformed chick embryo cells with a 1:20 dilution of antibody against CSP results in rapid blunting of cell processes, rounding, and increased detachment from the substratum, suggesting impaired cell adhesion.

Chick embryo cells transformed by the Schmidt-Ruppin strain of Rous sarcoma virus are shortened and rounded and fail to align in parallel arrays. CSP treatment results in flattening, elongation, and ultimately marked alignment of these cells (Fig. 1i and j). Similar changes occur when NRK cells transformed by Kirsten sarcoma virus are treated with CSP (Fig. 1k and l).

Two other transformants, NRK cells infected with Harvey sarcoma virus and L929 cells, become flattened with elongated processes but do not align as the cells crowd together (Fig. 1m and n). Two transformants that are already flattened, chick embryo fibroblasts transformed by Bryan high-titer Rous sarcoma virus and simian virus 40-transformed human WI38 cells, do not flatten further; instead their cell processes become more elongated. The two untransformed cell lines of epithelioid morphology, Balb/c 3T3 and NRK, do not flatten further, but instead display increased polarity and variable amounts of alignment (Fig. 1o and p).

Table 2. Cell-associated compared with substratum-associated labeled CSP

| Treatment | cpm* associated with cells | cpm* remaining on dish |
|---|---|---|
| Medium | 6 | 0 |
| + Buffer A | 15 | 16 |
| + CSP | 741 | 208 |
| + CSP, trypsinized | 9 | 28 |
| + CSP + anti-CSP | 884 | 165 |

CSP was prepared as described in *Materials and Methods* after labeling for 24 hr with 0.5 μCi/ml of [$^{14}$C]leucine (New England Nuclear, 324 mCi/mmol). SVT2 cells were plated in medium containing this labeled CSP at 50 μg/ml and fed at 24 hr. Controls received regular medium or the CSP diffusate. Two dishes were lightly trypsinized (2 μg/ml for 5 min) after 48 hr with labeled CSP. Two dishes were kept in labeled CSP plus a 1:20 dilution of the globulin fraction of anti-CSP for 48 hr. Control cultures showed no response, CSP cultures were highly elongated and aligned, and the cultures with anti-CSP showed a slight response. At 48 hr, cells and substratum-associated material were collected as described previously (4). After gel electrophoresis, the CSP bands (each piece 2.5 mm long) were cut out and eluted in 3% Protosol/97% Omnifluor. Total radioactivity per dish was determined; 2276 cpm of CSP were added at plating and again after 24 hr. Parallel gels were processed for autoradiography (6).

* Mean cpm in CSP per dish of duplicate dishes.

**CSP Is Adsorbed to the Cell Surface.** Previous experiments had shown that trace quantitites of isotopically labeled CSP were adsorbed intact onto the surface of nontransformed chick cells denuded of CSP (4). Likewise, intact CSP labeled with [$^{14}$C]leucine is present on monolayers of transformed SVT2 cells after culturing for 2 days with labeled CSP (Table 2); 16% of the labeled CSP added (half added at plating and half at feeding on day 1) remains associated with the cells after they are scraped from the dish; 5% remains on the dish. Autoradiography of the sodium dodecyl sulfate gels shows that the recovered labeled material has the same mobility as authentic CSP and that there are no significant labeled proteolytic breakdown products associated with the cells. After brief trypsinization (2 μg/ml for 5 min), over 98% of the cell-associated radioactive CSP is destroyed, indicating that the added CSP was on the cell surface and not ingested. Cultures treated with CSP plus anti-CSP still have at least as much labeled CSP bound to their surface (Table 2). We also quantitated the amount of CSP bound to the cells by densitometric scanning of the Coomassie blue stained gels and confirmed the results obtained with the radioactive method.

**CSP Increases Cell Spreading and Adhesion.** The area of SVT2 cells cultured for 2 days with CSP is increased 80% over controls (Table 3). The increase is not due to increased cell volume, since apparent mean cell volume increases only 21% (Table 3).

Cell-to-substratum adhesion was measured by the resistance of SVT2 cells to detachment from the substratum in calcium, magnesium-free medium. After vigorous shaking for 5 min, 25 times fewer cells are detached in cultures

*(Legend to Fig. 1 continued)*

Antagonism of CSP effects by anti-CSP (f, g, h). SVT2 cells cultured with CSP for 24 hr (f), with CSP plus a 1:15 dilution of anti-CSP (g), or the anti-CSP alone (h). Chick embryo fibroblasts transformed by the Schmidt-Ruppin strain of Rous sarcoma virus after 72 hr with buffer A (i) or CSP (j). NRK cells transformed by Kirsten sarcoma virus with buffer A (k) or CSP (l). NRK cells transformed by Harvey sarcoma virus after 48 hr with buffer A (m) or CSP (n). Balb/c 3T3 cells after 72 hr with buffer A (o) or CSP (p). All micrographs are 125×.

Table 3. Effects of CSP on SVT2 area and volume

| Treatment | Mean cell area ($\mu m^2$) | Mean cell volume ($\mu m^3$) |
|---|---|---|
| Medium | 242 | 1200 |
| + Buffer A | 246 | 1210 |
| + CSP | 442 | 1460 |

Cell area of fixed and stained cells was determined as described in *Materials and Methods*. For each point, $F$ = 15 fields, $I$ = 9000 points, and N = about 3000 cells. Cell volume was determined with a Coulter cell counter equipped with a Channelyzer attachment to obtain triplicate volume determinations of cells trypsinized after 3 days under the conditions indicated.

treated with CSP; the difference is still over 5-fold after 10 min (Table 4). This difference in adhesion to the substratum is apparent during routine feeding of dense cultures of SVT2 cells. Extreme care was necessary to prevent washing off the cells in control media; CSP-treated cultures were more firmly attached.

**CSP Restores a More Normal Nuclear Overlap Ratio.** The striking alignment of several cell types cultured with CSP suggested an increase in contact inhibition of movement (reviewed in ref. 10). A quantitative measure of contact inhibition of movement is provided by the overlap ratio, the ratio of observed overlapping of nuclei to the overlapping expected if there were no contact inhibition (i.e., a random distribution of nuclei).

Nuclear overlapping is reduced over 4-fold by CSP (Table 5). The high overlap ratios of the controls are accentuated by the presence of completely round cells, often located on top of other cells. If round cells are omitted, the mean values become: medium, 0.87 ± 0.05; buffer A, 1.13 ± 0.14; and CSP, 0.25 ± 0.03.

**CSP Does Not Restore Growth Control.** Culturing SVT2 cells in the presence of up to 100 $\mu$g/ml of CSP does not decrease their growth rate or their saturation density, as would have been expected if this protein plays a role in density-dependent inhibition of growth (Fig. 2). Control and CSP-treated SVT2 cells continue dividing until the cell layers slough from the dish. Likewise, added CSP does not inhibit the growth of NRK cells transformed by Kirsten or Harvey sarcoma viruses or by the Schmidt-Ruppin strain of Rous sarcoma virus (data not shown).

**The CSP Effects Are Not Due to Elevated Cyclic AMP.** Since cyclic AMP also affects cell shape and adhesiveness (11), we examined the effect of CSP on cyclic AMP levels. CSP does not increase cyclic AMP levels even in highly aligned SVT2 cells (Table 6). In fact, the levels of cyclic AMP are slightly decreased in CSP-treated cultures. Thus, CSP does not act through cyclic AMP. Furthermore, there are differences between the effect of these substances on

Table 4. Effect of CSP on SVT2 cell adhesion

| | 5 min | 10 min |
|---|---|---|
| Medium | 40.1 ± 8.9 | 53.4 ± 1.8 |
| + Buffer A | 36.3 ± 7.1 | 69.3 ± 5.3 |
| + CSP | 1.4 ± 1.1 | 10.3 ± 3.4 |

Mean ± standard deviation. Percent cell detachment after gyration on a rotary shaker was determined as described in *Materials and Methods*, using triplicate samples. Each CSP value differs from each of its controls at the $P < 0.005$ level of significance.

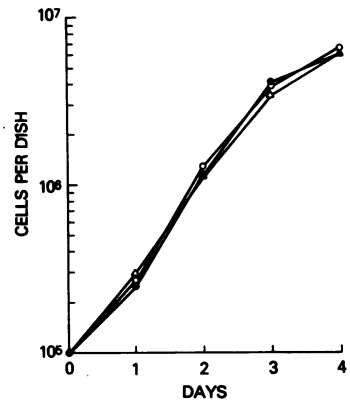


FIG. 2. Effect of CSP on cell growth. SVT2 cells were plated at $1 \times 10^5$ per 35 mm tissue culture dish in regular medium (●), medium plus 100 $\mu$l/ml of buffer A (○), or medium plus 100 $\mu$g/ml of CSP (△). At the times indicated, cultures were incubated with 0.25% trypsin, dissociated, and counted on a Coulter cell counter.

cultured cells. Cyclic AMP inhibits the growth of transformed rat and mouse cells whereas CSP does not. CSP affects the morphology of transformed chick embryo fibroblasts as well as Moloney sarcoma virus transformed NRK cells, whereas cyclic AMP has little effect on the morphology of these cells.

## DISCUSSION

Besides a decrease in CSP and other "LETS" proteins (see refs. 1–5), cell transformation results in many alterations in cell behavior. To determine which of these changes might be the result of losing CSP, we tested the effect of purified CSP on a variety of transformed cells. In general, this protein restores a more fibroblastic morphology, and in several cases it restores the alignment characteristic of confluent fibroblasts. The morphological effects of CSP that we report are of particular interest in that they occur in many transformants, and they result from the action of a single cellular macromolecular product at the cell surface.

Other treatments that result in flattening, elongation of cell processes, or increased alignment of certain transformed cells include cyclic AMP analogs, $N^6$ substituted adenines, butyrate (reviewed in ref. 11), galactose (12), plating onto monolayers of early passage mouse or hamster fibroblasts (13), conditioned medium from revertant melanoma cells (14), or material remaining on the substratum after removal of untransformed 3T3 cells (15). Although treatment with a cyclic AMP analogue does not promote CSP synthesis (unpublished data), some of the other treatments may do so. Further, a CSP-like molecule is present on the surface of early passage mouse and hamster embryo fibroblasts and on

Table 5. Effect of CSP on SVT2 nuclear overlap ratio

| | Overlap ratio |
|---|---|
| Medium | 1.16 ± 0.09 |
| + Buffer A | 1.41 ± 0.17* |
| + CSP | 0.26 ± 0.03† |

Means of overlap ratios ± standard error of mean; $n$ = 15 fields each, $b$ = 200 points per field; cell densities were $5 \times 10^4/cm^2$. The ratio of observed/expected nuclear overlaps was determined as described in *Materials and Methods*.

* Not significantly different from medium alone, $P = 0.2$.

† Significantly different from either control, both $P < 0.001$.

Table 6. Effect of CSP on intracellular cyclic AMP

| | Cyclic AMP (pmol/mg of protein) |
|---|---|
| Medium | 4.2 ± 0.2 |
| + Buffer A | 3.9 ± 0.1 |
| + CSP | 2.7 ± 0.2 |

Mean ± standard error of the mean of triplicate samples. SVT2 cells were plated in regular culture medium, then transferred to medium with or without 50 μg/ml of CSP or buffer A at 24 hr. Cultures were fed with these media again at 48 hr, and at 72 hr cyclic AMP was extracted and measured (16). Fig. 1c and d is photographs of the buffer A and CSP cultures.

the substratum after removal of 3T3 cells (unpublished data). Thus, these effects may be due to the presence of CSP.

Although the morphology of Schmidt-Ruppin Rous sarcoma virus-transformed chick cells after CSP treatment becomes similar to that of normal chick cells, none of the other treated transformants resembles their more epithelioid original parental cells (3T3 or NRK). Moreover, CSP-treated untransformed 3T3 and NRK cells become more elongated. We offer two explanations for these findings: (*a*) attaching CSP to any cell may tend to give it a more fibroblastic morphology or (*b*) the transformants are actually reverting to the more original mesenchymal cell phenotype.

Although the "LETS" class of proteins is thought to vary in accessibility to cell surface labeling in patterns consistent with a role in growth control (for references see 4 and 5), we cannot demonstrate inhibition of growth of several cell types at concentrations of CSP that result in striking morphological alterations. We can attach CSP to SVT2 cells so that CSP constitutes 2.7% of their total cell protein. CSP constitutes 2–3% of the cell protein of chick and mouse embryo cells (ref. 6; unpublished data). It therefore appears unlikely that the changes in CSP content in transformed cells are responsible for altered growth control. Since CSP readily attaches to SVT2 cells, and since no CSP breakdown products are seen on polyacrylamide gels of these treated cells, SVT2 cells probably are not producing large amounts of proteases. However, high protease activity might explain the reduced response of several transformants to CSP, although these cells might also be unable to bind CSP.

The simplest hypothesis to explain the effects of CSP on transformed cells is that this glycoprotein adheres directly to both cells and substrata. This property could explain the increased cell-to-substratum adhesion of CSP-treated cells, the cell spreading and flattening, and possibly the elongation of cell processes (if cell movement plus increased adhesion causes stretching of cell processes). In addition, increased cell-to-substratum or cell–cell adhesion could account for the restoration of a more normal overlap ratio by hindering the movement of a migrating cell under (or over) another cell. Culturing cells on substrata of lowered adhesiveness elevates overlap ratios (see ref. 10). Whether all of CSP's effects can be attributed to enhanced adhesiveness alone remains to be established. Our results do suggest that this major cell surface glycoprotein plays an important role in cell behavior *in vitro*.

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